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Effect of single pyrrole replacement with β-alanine on DNA binding affinity and sequence specificity of hairpin pyrrole/imidazole polyamides targeting 5′-GCGC-3′ ☆

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ABSTRACT

N-Methylpyrrole (Py)–*N*-methylimidazole (Im) polyamides are small organic molecules that can recognize predetermined DNA sequences with high sequence specificity. As many eukaryotic promoter regions contain highly GC-rich sequences, it is valuable to synthesize and characterize Py–Im polyamides that recognize GC-rich motifs. In this study, we synthesized four hairpin Py–Im polyamides **1**–**4**, which recognize 5′-GCGC-3′ and investigated their binding behavior with surface plasmon resonance assay. Py–Im polyamides **2**–**4** contain two, one, and one β-alanine units, replacing the Py units of **1**, respectively. The binding affinities of **2**–**4** to the target DNA increased 430, 390, and 610-fold, respectively, over that of **1**. The association and dissociation rates of **2** to the target DNA were improved by 11 and 37-fold, respectively, compared with those of **1**. Interestingly, the association and dissociation rates of **3** and **4** were higher than those of **2**, even though the binding affinities of **2**, **3**, and **4** to the target DNA were comparable to each other. The binding affinity of **2** to DNA with a 2 bp mismatch was reduced by 29-fold, compared with that to the matched DNA. Moreover, the binding affinities of **3** and **4** to the same mismatched DNA were reduced by 270 and 110-fold, respectively, indicating that **3** and **4** have greater specificities than **2** and are suitable as DNA-binding modules for engineered epigenetic regulation.

1. Introduction

N-Methylpyrrole (Py)–*N*-methylimidazole-(Im) polyamides are small molecules that bind to DNA with sequence-specificity and can be used as DNA-binding modules.[1–5] Py–Im polyamides can recognize specific DNA sequences in the minor groove of B-form DNA according to DNA recognition rules.[6,7] Py favors T, A and C bases, excluding G; Im is a G-reader. The lone electron pair of N3 in Im forms a hydrogen bond with the 2-amino hydrogen of guanine. Anti-parallel pairing of Im/Py specifies G·C, whereas Py/Im specifies C·G. Anti-parallel pairing of Py/Py specifies A·T or T·A degenerately.[6,7]

Recently, we measured the K_Ds, k_as and k_ds of Py–Im polyamides with different numbers of Im rings and these data indicated that the association rate of the Py–Im polyamides with their target DNA decreased as the number of Im in the Py–Im polyamides increased, even though their dissociation rates were comparable with each other.[8] We calculated the model structures of four-ring Py–Im polyamides by density functional theory.[8] These data suggested that an increase in planarity of Py–Im polyamide induced by the incorporation of Im reduced the association rate of Py–Im polyamides.

Many eukaryotic genes contain highly GC-rich sequences in the respective promoter regions.[9–12] One representative DNA-binding domain that binds to GC-rich sequences is a zinc-finger, common among many transcription factors.[13] As Py–Im polyamides can bind to DNA with sequence specificity comparable to DNA-binding proteins, it is potentially useful to synthesize Py–Im polyamides that recognize GC-rich promoter regions and thereby regulate gene expression. However, as described above, GC-rich sequences such as 5′-GCGC-3′ and 5′-CGCG-3′ have been identified as difficult recognition sequences for Py–Im polyamides due to the aforementioned planarity of Im-containing polyamides.[8,14,15]

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Interestingly, as reported previously, replacement of two Py with aliphatic β-alanine can increase binding affinity and provide flexibility in the polyamide structures. The binding affinity of Im-β–ImPy-γ–Im-β–ImPy-β–Dp, which recognizes 5′-GCGC-3′, was 100-fold greater than that of ImPyImPy-γ–ImPyImPy-β–Dp[14] (β = β-alanine, γ = γ-aminobutyric acid, and Dp = ((dimethylamino)propyl)amide). However, the corresponding association rate constant and dissociation rate constant have not determined. Furthermore, we lack information about the Py–Im polyamides containing only one replacement of Py with β-alanine. As design of Py–Im polyamides with higher DNA binding affinities and sequence specificities is crucial for the production of synthetic DNA binding modules, the detail molecular characterization of Py replacement with β-alanine is valuable not only from a practical stand point, but also for the elucidation of the DNA recognition mechanism of Py–Im polyamides. In this study, we synthesized four hairpin Py–Im Polyamides **1**–**4** that recognize Py–Im polyamide **2** contains two pairs of Im/β, and **3** and **4** each contain one Im/β pair. We measured the K_Ds, k_as and k_ds of the Py–Im polyamide to matched and mismatched binding sites by surface plasmon resonance (SPR) assay. Our SPR data suggest that one Py-replacement with β is sufficient to increase the binding affinity of the Py–Im polyamide targeting 5′-GCGC-3′. However, the k_as and k_ds of **3** and **4** to the matched DNA were higher than those of **2**. We also measured the DNA binding affinity of **1**–**4** to various mismatched DNA targets to determine their sequence specificities and these data provide valuable information for the design of Py–Im polyamides that recognize GC-rich sequences, and elucidate the target DNA recognition mechanism.

2. Results

2.1. Hairpin Py–Im polyamide synthesis and DNA preparation

As reported previously, our SPR data and calculated model structures of 4-ring Py–Im polyamides suggested that an increase in planarity of the Py–Im polyamide induced by the incorporation of Im reduced the association rate of Py–Im polyamides.[8] However, two replacements of Py by β in the hairpin Py–Im polyamide that recognizes 5′-GCGC-3′ can increase the binding affinity.[14] In this study, to characterize the effect of Py replacement with β in more detail, we synthesized four hairpin Py–Im polyamides **1**–**4** (Fig. 1) by the Fmoc-chemistry solid-phase synthesis method. Py–Im polyamide **1**, **2**, **3**, and **4** contain zero, two, one and one internal Im/β pairs, respectively. As shown in Figure 1, Py–Im polyamide **2** contains two Im-β-Im, and **3** and **4** contain one Im-β-Im at the C-terminal side and N-terminal side, respectively. Based on the recognition rule of Py–Im polyamides, the target DNA sequence of **1**–**4** is WGCGCW,[6,7] however, because of symmetry of the target DNA sequences, two single-mismatched target DNA sequences, WGNGCW and WGCNCW are not distinguishable. Previously, we synthesized five Py–Im polyamides containing two βs at the N-terminal and demonstrated that the β-Dp at the C-terminal of the Py–Im polyamides had a slight steric preference for A·T or T·A relative to G·C or C·G.[8] Therefore, in this study, we synthesized the aforementioned four hairpin Py–Im polyamides **1**–**4** containing two βs at N-terminus, and prepared four hairpin DNAs, ODN1–4 to characterize the binding affinity of the Py–Im polyamides containing Im/β pairs and the relationship between the position of the β substitution for Py and a mismatched DNA site (Fig. 1). ODN2 and 3 contains one mismatched DNA site and ODN4 contains two mismatched DNA sites (Fig. 1b). After synthesis of **1**–**4**, we purified them by reverse phase HPLC and confirmed that the purity of **1**–**4** were more than 95% by analytical HPLC and ESI-TOFMS.

2.2. Aliphatic β-alanine increases the DNA association rate constant and decreases the DNA dissociation rate constant

To characterize the DNA binding affinity of **1**–**4**, the SPR experiments were performed as described in the experimental section and the respective dissociation equilibrium constant (K_D), association rate constant (k_a) and dissociation rate constant (k_d) of **1**–**4** against the target DNAs were measured. ODN1, which includes the target DNA sequence of **1**–**4**, was immobilized to a streptavidin-coated sensor chip and the Py–Im polyamide solutions were injected. As shown in Figure 2, the SPR sensorgrams were obtained, and kinetic binding parameters K_D, k_a and k_d were determined (Table 1). The K_D, k_a and k_d of **1** were 2.7×10^{-7} M, 8.2×10^4 M^{-1} s^{-1}, and 0.022 s^{-1}, respectively, and these data were consistent with previous data.[8] The K_D of **2** was determined to be 6.3×10^{-10} M, and the binding affinity of **2** was 430-fold over that of **1**, which is consistent with previous data.[14] The k_a and k_d of **2** were determined to be 9.4×10^5 M^{-1} s^{-1} and 5.9×10^{-4} s^{-1}, respectively, and interestingly, the k_a and k_d of **2** were improved by 11 and 37-fold, respectively, compared with those of **1**.

In this study, we also measured the K_D, k_a and k_d of **3** and **4** (Fig. 2c and d, and Table 1). The K_Ds of **3** and **4** were determined to be 7.0×10^{-10} and 4.4×10^{-10} M, respectively. The binding affinities of **3** and **4** were 390 and 610-fold, respectively, over that of **1**, and comparable to that of **2**. However, the k_as of **3** and **4** were determined to be 1.3×10^7 and 1.5×10^7 M^{-1} s^{-1}, respectively, and the k_ds of **3** and **4** were determined to be 9.1×10^{-3} and 6.5×10^{-3} s^{-1}, respectively (Table 1). Compared with those of **2**, the k_as of **3** and **4** were increased by 14 and 16-fold, however, the k_as of **3** and **4** were also increased by 15 and 11-fold, respectively, indicating that **3** and **4** bind to and dissociate from the target DNA ODN1 faster than **2**.

2.3. DNA binding affinities of Py–Im polyamide 1–4 to mismatched DNA

As described above, we demonstrated that a single Py replacement with β can increase the binding affinity of the Py–Im polyamide just as well as two replacements with β. Interestingly, even though the K_Ds of **3** and **4** were comparable to that of **2**, the k_as and k_ds of **3** and **4** were higher than those of **2**. These results indicated that the properties of **3** and **4** are different from **2**, and further characterizations of these Py–Im polyamides would clarify not only the effect of Py-replacement with β-alanine, but also the target DNA recognition mechanism of Py–Im polyamide. Therefore, we measured the K_D, k_a and k_d of **1**, **2**, **3** and **4** using ODN2, 3 and 4 (Supplementary Figs. S1–S3 and Tables 2–4) to characterize the sequence-specificity of **1**–**4**. The binding affinities of **1** to ODN2, 3 and 4 were significantly decreased by 250, 230 and 300-fold, respectively, compared with that of ODN1. On the other hand, the binding affinities of **2** to ODN2, 3 and 4 were decreased by 17, 17 and 29-fold, respectively, compared with that of ODN1 (Fig. 2 and Supplementary Figs. S1–S3 and Table 1–4). The k_as and k_ds of **2** to ODN2, 3 and 4 were impaired moderately by 2.2 to 11-fold, compared with those of ODN1 (Tables 1–4). By contrast, the binding affinities of **1** to ODN2, 3 and 4 were significantly reduced, and especially the k_as were reduced by 40 to 60-fold, compared to ODN1 (Tables 1–4).

In this study, we also determined the K_D, k_a and k_d of **3** and **4** using ODN2, 3 and 4 (Supplementary Figs. S1–S3 and Tables 2–4). The binding affinities of **3** to ODN2, 3 and 4 were 1.1×10^{-8}, 8.3×10^{-8} and 1.9×10^{-7} M, respectively (Tables 2–4). Interestingly, the binding affinity of **3** to ODN2 was reduced by 16-fold relative to ODN1, which was comparable to those of **2** to ODN2, 3 or 4; however, the binding affinities of **3** to ODN3 or 4 were significantly reduced by 120 or 270-fold, respectively. Regarding Py–Im

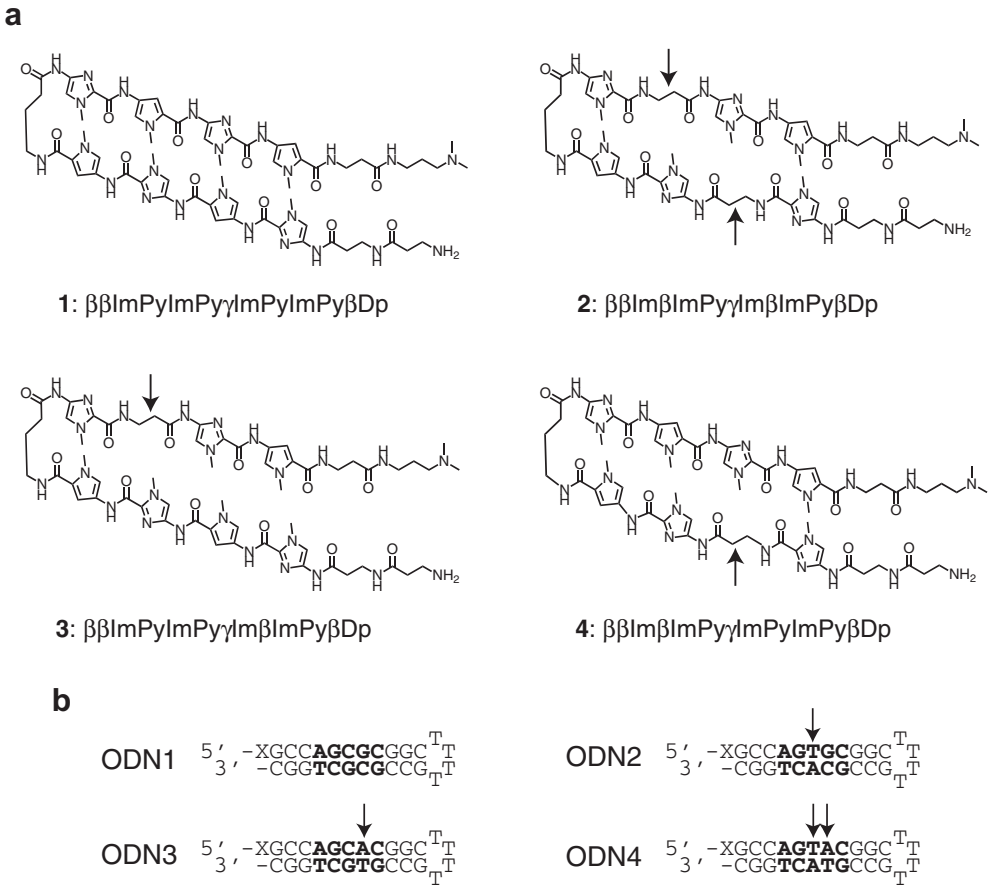


Figure 1. (a) Structures of Py–Im polyamides **1**–**4**. The arrows indicate the positions of replacement of Py with β. (b) Sequence of 5′-biotinylated hairpin DNA (ODN1-4). X represents biotin. The binding sequences of the Py–Im polyamides are shown in bold. The arrows indicate the mismatch-recognition bases.

polyamide **4**, the binding affinities of **4** to ODN2, 3 and 4 were 8.9×10^{-8}, 1.1×10^{-8} and 4.7×10^{-8} M, respectively (Tables 2–4), and the binding affinity of **4** to ODN3 was reduced by a rather modest 25-fold, comparable to that of **3** to ODN2. However, the binding affinities of **4** to ODN2 or 4 were as significantly reduced as those of **3** to ODN3 or 4.

3. Discussion

3.1. DNA binding affinities to the target DNA

In this study, to characterize substitution of β for Py, especially the difference between two β substitutions and a single replacement of Py with β in hairpin Py–Im polyamide targeting 5′-GCGC-3′, we measured the K_Ds, k_as and k_ds of four hairpin Py–Im Polyamides **1**–**4** to matched target or mismatched DNAs. The K_D of **2** was 430-fold over that of **1**, and the k_a and k_d of **2** were improved by 11 and 37-fold, respectively, compared with those of **1**. Previously, Dervan and co-workers have also reported the NMR structures of Py–Im polyamide containing β (Im-β–Im and Py-β–Im) bound with the target DNA, and the dihedral angles of the two Im rings in Im-β–Im, and between Py ring and Im ring in Py-β–Im, were determined to be 50° and 33°, respectively.[16] They suggested that the increase in dihedral angle for Im-β–Im is due to the need for proper orientation to maximize hydrogen bonding between N3 in Im and the 2 amino hydrogen of G.[16] In this study, we showed that the k_a and k_d of **2** were improved significantly, compared with those of **1**. These results suggest that structural flexibility of **2** by replacement of two Py with β results in more stable complex formation of **2**/ODN1 compared with **1**, and that it requires less energy to change the structure for binding to the target DNA.

The K_Ds of **2**, **3** and **4** to ODN1 were comparable to each other, however, interestingly, the k_as and the k_ds of **3** and **4** were higher than those of **2**. To obtain insight into the difference among the observed k_a and k_d values, we calculated the model structure of ImβImPy by Density Functional Theory, without the target DNA. The structure was folded (data not shown), but it was not curved like the model structure of ImPyImPy reported previously,[8] suggesting that ImβImPy is considerably more flexible compared with ImPyImPy, and may form an unfavorable folded structure for DNA binding. However, ImPyImPy in **3** and **4** may restrict the flexibility of ImβImPy, and suppress the unfavorable folded structure for DNA binding, resulting in the observed higher k_as of **3**/ODN1 and **4**/ODN1, compared with that of **2**. However, because **3** and **4** contain one ImPyImPy, stabilities of **3**/ODN1 and **4**/ODN1 are less than that of **2**/ODN1, resulting in higher k_ds of **3**/ODN1 and **4**/ODN1, compared with that of **2**.

3.2. Application of the Py–Im polyamides to DNA binding modules for genetic regulation

Because Py–Im polyamides conjugated with chromatin-modifying suberoylanilide hydroxamic acid (SAHA) have been recently used as artificial transcriptional activators for epigenetic regulation,[17–19] not only higher DNA binding affinity but also stronger sequence specificity are crucial for the design of the Py–Im polyamides to suppress nonspecific DNA binding of the designed

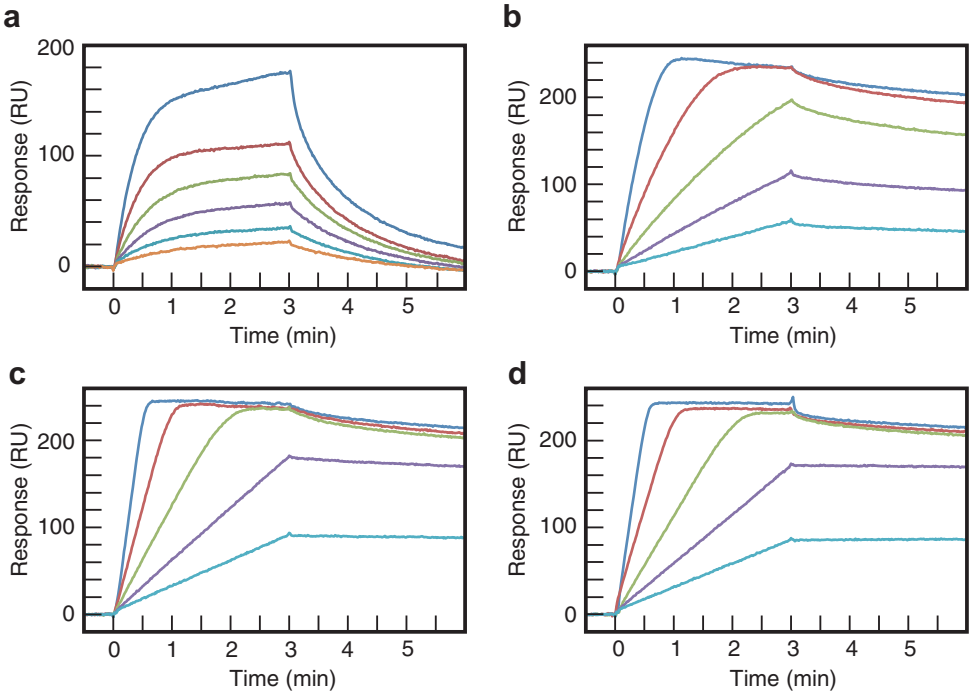


Figure 2. SPR sensorgrams for the interaction of Py–Im polyamides with ODN1 immobilized on a sensor chip SA. (a) Py–Im polyamide **1** at concentrations of 25 (orange, lowest curve), 50 (light blue), 100 (purple), 200 (green), 400 (red), and 800 nM (blue, highest curve). (b) Py–Im polyamide **2** at concentrations of 12.5 (light blue, lowest curve), 25 (purple), 50 (green), 100 (red), and 200 nM (blue, highest curve). (c) Py–Im polyamide **3** at concentrations of 12.5 (light blue, lowest curve), 25 (purple), 50 (green), 100 (red), and 200 nM (blue, highest curve). (d) Py–Im polyamide **4** at concentrations of 12.5 (light blue, lowest curve), 25 (purple), 50 (green), 100 (red), and 200 nM (blue, highest curve).

Table 1
Binding affinity of **1**–**4** with ODN1

Py–Im polyamide[a]	K_D (10^{-10} M)	k_a (10^4 M^{-1} s^{-1})	k_d (10^{-4} s^{-1})
	2700 ± 520	8.2 ± 4.2	220 ± 63
	6.3 ± 4.3	94 ± 32	5.9 ± 2.0
	7.0 ± 5.4	1300 ± 1000	91 ± 40
	4.4 ± 2.2	1500 ± 20	65 ± 32

[a] Closed circle, open circle, and β indicate Im, Py, and β-alanine, respectively.

Table 2
Binding Affinity of **1**–**4** with ODN2

Py–Im polyamide[a]	K_D (10^{-8} M)	k_a (10^3 M^{-1} s^{-1})	k_d (10^{-3} s^{-1})
	6800 ± 880	1.9 ± 0.33	130 ± 20
	1.1 ± 0.18	250 ± 22	2.8 ± 0.27
	1.1 ± 0.20	3700 ± 3000	43 ± 39
	8.9 ± 1.5	160 ± 34	14 ± 0.14

[a] Closed circle, open circle, and β indicate Im, Py, and β-alanine, respectively.

Table 3
Binding affinity of **1**–**4** with ODN3

Py–Im polyamide[a]	K_D (10^{-8} M)	k_a (10^3 M^{-1} s^{-1})	k_d (10^{-3} s^{-1})
	6300 ± 3000	1.2 ± 0.34	79 ± 62
	1.1 ± 0.32	360 ± 210	3.4 ± 1.2
	8.3 ± 0.64	1300 ± 340	112 ± 37
	1.1 ± 0.52	2500 ± 1200	25 ± 0.25

[a] Closed circle, open circle, and β indicate Im, Py, and β-alanine, respectively.

Table 4
Binding affinity of **1**–**4** with ODN4

Py–Im polyamide[a]	K_D (10^{-8} M)	k_a (10^3 M^{-1} s^{-1})	k_d (10^{-3} s^{-1})
	8200 ± 6500	1.2 ± 1.2	100 ± 40
	1.8 ± 0.65	420 ± 170	6.8 ± 0.61
	19 ± 6.1	240 ± 100	41 ± 9.6
	4.7 ± 0.11	830 ± 260	39 ± 13

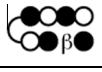
[a] Closed circle, open circle, and β indicate Im, Py, and β-alanine, respectively.

Py–Im polyamides. As described above, the binding affinities of **2**, **3** and **4** to the matched DNA were comparable to each other. In this study, we also measured the K_Ds, k_as and k_ds of **1**, **2**, **3**, and **4** to ODN2, 3, and 4, which contained 1 bp or 2 bp mismatches compared to the matched target DNA, and determined the sequence specificities of **1**–**4** (Supplementary Figs. S1–S3 and Tables 2–4).

The K_Ds of **1** to ODN2, 3 and 4 were decreased by 250, 230 and 300-fold, respectively, compared with that of ODN1 and the k_as in particular were reduced by 40 to 60-fold (Tables 1–4), whereas the K_Ds of **2** to ODN2, 3 and 4 were decreased by 17, 17 and 29-fold, respectively, compared with ODN1 (Tables 1–4). To evaluate sequence specificities of **2**–**4**, we also determined the free energy change (ΔG^0, kcal/mol) from the K_D upon the formation of the Py–Im polyamides **1**–**4**/DNA complexes (Table 5). The differences in ΔG^0 between **1**/ODN1 and **1**/ODN2, **1**/ODN3, or **1**/ODN4 were −3.3, −3.3, and −3.4 kcal/mol, respectively, (Table 5), whereas the differences in ΔG^0 between **2**/ODN1 and **2**/ODN2, **2**/ODN3, or **2**/ODN4 were −1.8, −1.7, and −2.1 kcal/mol, respectively (Table 5). These results suggest that due to the flexibility of the two ImβImPy at the N- and C-terminal sides in **2**, the structure of **2** is able to change easily and the curvature of **2** may precisely match the minor groove of 1 bp or 2 bp mismatched DNA sites, resulting in only a moderate decrease of the k_as and k_ds of **2** to ODN2, 3 and 4, compared with those for ODN1. On the other hand, due to the planar structure and the lack of the structural flexibility of **1**, the structure of **1** is not able to change as much as **2**, therefore, the k_as of **1** to mismatched DNA targets were significantly reduced, even to a 1 bp mismatched DNA target such as ODN2 or 3. Thus, even though the DNA binding affinity of **1** is much lower than that of **2**, **1** has a stronger sequence specificity than **2**.

In the case of **3** and **4**, interestingly, the differences in ΔG^0 between **3**/ODN1 and **3**/ODN2 and between **4**/ODN1 and **4**/ODN3 were −1.5 and −2.0 kcal/mol (Table 5), respectively, indicating that the binding affinities of **3**/ODN2 and **4**/ODN3 were comparable to those of **2**/ODN2, 3, and 4. However, as discussed below, these are exceptional sequence specificities. On the other hand, the differences in ΔG^0 between **3**/ODN1 and **3**/ODN3 or **3**/ODN4, or between **4**/ODN1 and **4**/ODN2 or **4**/ODN4 were −2.7 ≈ −3.2 kcal/mol (Table 5), indicating that **3** and **4** have similar sequence specificities to **1**. Furthermore, Dervan and co-worker have demonstrated that the binding affinities of the Py–Im polyamide, Im-β-ImPy-γ–Im-β-ImPy-β–Dp, corresponding to **2** in this study, for 5′-TGGCCA-3′ and 5′-TGGGGA-3′ were decreased by 26 and 34-fold, compared with the binding affinity for 5′-TGCGCA-3′,[14] which is consistent with our data, as described above. These results indicate that the DNA binding affinities of **2**, **3**, and **4** were comparable to each other, but, **3** and **4** have stronger sequence specificities than **2**. Thus, rather than Py–Im polyamide **2**, Py–Im polyamide **3** and **4** are suitable for DNA binding modules for epigenetic regulation.

Table 5
Free energy change (ΔG^{0a}, kcal/mol) on the formation of **1**–**4**/ODN1–4 complexes

Py–Im polyamide[b]	ODN1 5′-AGCGC-3′	ODN2 5′-AGCGC-3′	ODN3 5′-AGCGC-3′	ODN4 5′-AGCGC-3′
	−9.0	−5.7 (3.3)[c]	−5.7 (3.3)	−5.6 (3.4)
	−12.6	−10.8 (1.8)	−10.9 (1.7)	−10.5 (2.1)
	−12.4	−10.9 (1.5)	−9.7 (2.7)	−9.2 (3.2)
	−12.8	−9.6 (3.2)	−10.8 (2.0)	−10.0 (2.8)

[a] ΔG^0 values are calculated from the equation, $\Delta G^0 = -RT \ln 1/KD$, where R is the universal gas constant, 1.987 cal/mol K; T is absolute temperature in K, here 298.15 K.
[b] Closed circle, open circle, and β indicate Im, Py, and β-alanine, respectively.
[c] The numbers in brackets indicate the discrimination energy, which is defined as the difference of the free energy of each Py–Im polyamide/mismatched DNA complex from that of matched DNA.

3.3. Polyamide polymers at the N-terminal and C-terminal sides in hairpin Py–Im polyamide assist and coordinate each other for matched and mismatched DNA binding

As described above, the K_Ds of **3** and **4** to the matched DNA were comparable to that of **2**, whereas the k_as and k_ds of **3** and **4** were higher than those of **2**, indicating that further characterizations of these Py–Im polyamides would clarify not only the effect of Py replacement with β, but also the detailed target DNA recognition mechanism of Py–Im polyamide.

Our SPR data showed that the binding affinities of **3**/ODN2 and **4**/ODN3 were comparable to those of **2**/ODN2, 3, and 4, whereas the binding affinities of **3**/ODN3, **3**/ODN4, **4**/ODN2, and **4**/ODN4 were as significantly reduced as those of **1**/ODN2, 3, and 4. Because ImβImPy in **3** is located at the top strand of ODN2, 5′-GTGC-3′ and ImβImPy in **4** is located at the bottom strand of ODN3, 5′-GTGC-3′, the ImβImPy interacts with 5′-GTGC-3′ precisely. By contrast, ImPyImPy in **3** and **4** is placed at 5′-GCAC-3′ and the Im located at the third position from the N-terminus faces a mismatched A in 5′-GCAC-3′. Because of the structural flexibility of the ImβImPy, the precise interaction between the ImβImPy and 5′-GTGC-3′ may assist the unstable interaction between ImPyImPy and 5′-GCAC-3′ to form the complex of **3**/ODN2 or **4**/ODN3, resulting in moderately impaired binding affinities for **3**/ODN2 and **4**/ODN3, compared with those of **3**/ODN3 and **4**/ODN2.

In contradiction to **3**/ODN2 or **4**/ODN3, in the case of **3**/ODN3 and **4**/ODN2 the ImβImPy is placed at the 1 nt mismatched 5′-GCAC-3′ and the ImPyImPy is placed at the matched 5′-GTGC-3′, and the binding affinities of **3**/ODN3 and **4**/ODN2 were significantly reduced. In the case of **3**/ODN4 and **4**/ODN4, their binding affinities were comparable to those of **3**/ODN3 or **4**/ODN2, and interestingly, these affinities were better than that of **1**/ODN1 (Tables 1–3). However, because the complex of the ImβImPy with the 1 nt mismatched DNA site is less stable than it is with a matched DNA site, the binding affinities of **3**/ODN3, **3**/ODN4, **4**/ODN2, or **4**/ODN4 were reduced, compared with those of **3**/ODN2 or **4**/ODN3.

As described above, the binding affinities of **2** to ODN2, 3 and 4 were comparable to each other. Because **2** contains two ImβImPys at the N-terminal and C-terminal sides, in the case of ODN4, both the ImβImPys face the 1 nt mismatched DNA, 5′-GTAC-3′; however, the binding affinity of **2**/ODN4 was comparable to those of **2**/ODN2 or 3. Furthermore, as discussed above, Dervan and co-workers have demonstrated that the binding affinities of the Py–Im polyamide, Im-β–ImPy-γ–Im-β–ImPy-β–Dp, for 2 bp mismatched DNA targets, 5′-TGGCCA-3′ and 5′-TGGGGA-3′, were decreased by 26 and 34-fold, compared with the affinity for matched DNA 5′-TGCGCA-3′.[14] In the case of 5′-TGGCCA-3′, both the ImβImPys face 2 nt mismatched DNA, 5′-GGCC-3′. In the case of 5′-TGGGGA-3′, one ImβImPy faces 2 nt mismatched DNA, 5′-GGGG-3′, while another ImβImPy faces a 2 nt mismatched DNA, 5′-CCCC-3′. These results suggest that even though both ImβImPys face 2 nt mismatched DNAs, due to the flexibility of ImβImPy, both ImβImPys manage to interact with the mismatched DNA, and the N-terminal and C-terminal polyamides in the Py–Im polyamide assist each other for binding to the target DNA.

In this study, using SPR assay, we demonstrated the effect of replacements of Py with β on the improvement of the association and dissociation rate constants, and the sequence specificity of the Py–Im polyamide. Even though the target DNA sequence of the Py–Im polyamide is determined by the anti-parallel pairings of the Py–Im polyamide,[6,7] our data suggest that structural flexibilities of the N-terminus and C-terminus of the polyamides are substantially involved in the association and dissociation rate constants of the respective Py–Im polyamide, and consequently the binding affinity of the Py–Im polyamide to mismatched DNA.

We are now planning to synthesize Py–Im polyamides with different numbers of Im and substitution of β for Py. Further analysis of these Py–Im polyamides will provide valuable information about the effect of β substitution and the target DNA recognition mechanism.

4. Experimental

4.1. General

The following abbreviations were used: Fmoc, fluorenylmethoxycarbonyl; DMSO, dimethyl sulfoxide; TFA, trifluoroacetic acid; β, β-alanine; γ, γ-aminobutyric acid; Py, N-methylpyrrole; Im; N-methylimidazole, Dp; 3-(dimethylamino)propylamine.

Electrospray ionization time-of-flight mass spectrometry (ESI-TOFMS) was conducted with a BioTOF II (Bruker Daltonics) mass spectrometer to determine the molecular weight of Py–Im polyamides **1**–**4**.

4.2. Polyamide synthesis

Py–Im polyamides **1**–**4** were synthesized in a stepwise reaction using a previously described Fmoc solid-phase protocol.[15] Syntheses were performed using a Pioneer Peptide Synthesizer (PSSM-8, Shimadzu) with a computer-assisted operation system on a 36 μmol scale (100 mg of Fmoc-β-alanine Wang resin). After the synthesis, Dp was mixed with the resin and the mixture was shaken at 550 rpm for 4 h at 55 °C to detach the Py–Im polyamides from the resin. Purification of Py–Im polyamides **1**–**4** were performed using a high-performance liquid chromatography (HPLC) PU-2080 Plus series system (JASCO), using a 10 mm × 150 mm ChemcoPak Chemcobond 5-ODS-H reverse-phase column in 0.1% TFA in water with acetonitrile as eluent, at a flow rate of 3 mL/min, and a linear gradient from 20% to 60% acetonitrile over 20 min, with detection at 254 nm. Collected fractions were analyzed by ESI-TOFMS.

4.3. Surface plasmon resonance (SPR) assay

All SPR experiments were performed on a BIACORE X instrument at 25 °C as described previously.[15,20] The sequences of biotinylated hairpin DNAs containing target sequences are shown in Figure 1b. The hairpin DNAs were immobilized on a streptavidin-coated SA sensor chip at a flow rate of 20 μL/min to obtain the required immobilization level (up to approximately 1400 resonance units (RU) rise). Experiments were carried out using HBS-EP buffer (10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 150 mM NaCl, 3 mM ethylenediaminetetraacetic acid (EDTA), and 0.005% Surfactant P20) with 0.1% DMSO at 25 °C, pH 7.4. A series of sample solutions were prepared in HBS-EP buffer with 0.1% DMSO and injected at a flow rate of 20 μL/min. To measure association and dissociation rate constants (K_D, k_a and k_d), data processing was performed with an appropriate fitting model using the BIAevaluation 4.1 program. The sensorgrams of all data were fitted by using the 1:1 binding model with mass transfer. The values of K_D, k_a, and k_d for all data are summarized in Tables 1–4.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2013.06.005.

References and notes

1. Gottesfeld, J. M.; Neely, L.; Trauger, J. W.; Baird, E. E.; Dervan, P. B. *Nature* **1997**, *387*, 202.
2. Mapp, A. K.; Ansari, A. Z.; Ptashne, M.; Dervan, P. B. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 3930.
3. Murty, M. S.; Sugiyama, H. *Biol. Pharm. Bull.* **2004**, *27*, 468.
4. Kwon, Y.; Arndt, H. D.; Mao, Q.; Choi, Y.; Kawazoe, Y.; Dervan, P. B.; Uesugi, M. *J. Am. Chem. Soc.* **2004**, *126*, 15940.
5. Nickols, N. G.; Dervan, P. B. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 10418.
6. Dervan, P. B.; Edelson, B. S. *Curr. Opin. Struct. Biol.* **2003**, *13*, 284.
7. Dervan, P. B.; Poulin-Kerstien, A. T.; Fechter, E. J.; Edelson, B. S. *Top. Curr. Chem.* **2005**, *253*, 1.
8. Han, Y. W.; Matsumoto, T.; Yokota, H.; Kashiwazaki, G.; Morinaga, H.; Hashiya, K.; Bando, T.; Harada, Y.; Sugiyama, H. *Nucleic Acids Res.* **2012**, *40*, 11510.
9. Bird, A. P. *Trends Genet.* **1987**, *3*, 342.
10. Antequera, F.; Bird, A. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 11995.
11. Song, J.; Ugai, H.; Kanazawa, I.; Sun, K.; Yokoyama, K. K. *J. Biol. Chem.* **2001**, *276*, 19897.
12. Saxonov, S.; Berg, P.; Brutlag, D. L. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 1412.
13. Kuwahara, J.; Yonezawa, A.; Futamura, M.; Sugiura, Y. *Biochemistry* **1993**, *32*, 5994.
14. Turner, J. M.; Swalley, S. E.; Baird, E. E.; Dervan, P. B. *J. Am. Chem. Soc.* **1998**, *120*, 6219.
15. Minoshima, M.; Bando, T.; Sasaki, S.; Fujimoto, J.; Sugiyama, H. *Nucleic Acids Res.* **2008**, *36*, 2889.
16. Urbach, A. R.; Love, J. J.; Ross, S. A.; Dervan, P. B. *J. Mol. Biol.* **2002**, *320*, 55.
17. Pandian, G. N.; Nakano, Y.; Sato, S.; Morinaga, H.; Bando, T.; Nagase, H.; Sugiyama, H. *Sci. Rep.* **2012**, *2*, 544.
18. Pandian, G. N.; Sugiyama, H. *Biotechnol. J.* **2012**, *7*, 798.
19. Pandian, G. N.; Ohtsuki, A.; Bando, T.; Sato, S.; Hashiya, K.; Sugiyama, H. *Bioorg. Med. Chem.* **2012**, *20*, 2656.
20. Zhang, W.; Bando, T.; Sugiyama, H. *J. Am. Chem. Soc.* **2006**, *128*, 8766.